IgG SUBCLASS DEFICIENCY

IN

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ABSTRACT

The incidence of IgG subclass deficiency has been well documented in the Western World. In a study of 8015 healthy blood donors, 4% were found to be deficient in IgG2 subclass. No local information is available on this disease entity. IgG subclass deficiency though presented with recurrent sinopulmonary infections and otitis media, can easily escape the first line clinical and laboratory investigation especially in the health care system in Hong Kong when patients are not cared by their family doctor.

The Beckman Array 360 Protein System is an analyser utilising the principle of rate nephelometry to quantify protein in biological fluid. The Binding Site IgG Subclass Array Kit has been optimised for the Array 360 Protein System. A study was conducted to evaluate the performance of The Binding Site IgG subclass assays on the Array 360 Protein System. A pilot study was conducted on 53 patients with recurrent infections, bronchiectasis and otitis media together with 26 control subjects.

Evaluation of the IgG subclass assays demonstrated within-batch CV of 0.6% to 1.9% and inter-assay CV of 1.4% to 4.3%. Lowest limits of detection for the four subclasses are 1494mg/L, 318mg/L, 108mg/L and 132mg/L for IgG1, IgG2, IgG3 and IgG4 respectively. These performance characteristics are within that claimed by The Binding Site. The assays are easy to perform and a panel of four IgG subclasses can be performed in approximately three minutes once the instrument is running.

The laboratory study did not reveal any case of IgG subclass deficiency in the patient group. IgG1 and IgG3 of patients were significantly higher than that of the control group by Mann Whitney U test with both p values of <0.005. Therefore in the context of IgG subclass deficiency, there was no difference between the patient group and control group. IgG2 levels of patient with bronchiectasis also showed no difference when compared with the control group.

摘 要

免疫球蛋白G可再細分爲四個亞類。 西方國家對免疫球蛋白G亞類缺乏已有詳細的記載。 其中一個研究曾發現在8015名身體健康的捐血者之中有高達4%爲免疫球蛋白G亞類二型缺乏。 在香港則未有這方面的數據。 復發性鼻竇, 呼吸道感染及中耳炎爲免疫球蛋白G亞類缺乏的一些症狀。 這些感染也會輕易被忽視爲普通的感染。

貝克曼儀器公司的"Array 360 Protein System"是一部以速率散射法去測定血漿蛋白濃度的機器。 這個研究以"The Binding Site"公司生產的免疫球蛋白G亞類試劑用於"Array 360 Protein System"對53名患有復發性感染, 支氣管擴張, 中耳炎等病症的病人及一個26人的對照組進行。

免疫球蛋白G亞類四種試劑的檢定得出同批精密度由0.6%到1.9%, 批間精密度則由1.4%到4.3%。 四種免疫球蛋白G亞類的最低檢測限制分別爲1494mg/L, 318mg/L, 108mg/L 及 132mg/L。 這些數據和"The Binding Site"所提供的表現指標相約。 免疫球蛋白G亞類試劑配合"Array 360 Protein System"可於機器開始運行後每三分鐘就能測定一套四個免疫球蛋白G亞類的濃度, 從而提供了一個快捷的自動化測試方法。

在本研究中並未發現有病人爲免疫球蛋白G亞類缺乏。 病人組的免疫球蛋白G亞類一型及三型的濃度顯著高於對照組($p<0.005$)。 曾有報告指出支氣管擴張病患者中部份會有免疫球蛋白G亞類二型缺乏, 在本研究中沒有同樣的發現。

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CHAPTER 1
INTRODUCTION

1.1 Overview

Five major classes of immunoglobulins are normally present in blood. Clonal expansion of B-lymphocytes, upon recognition of a foreign antigen, differentiates into plasma cells. Immunoglobulins are produced by plasma cells.

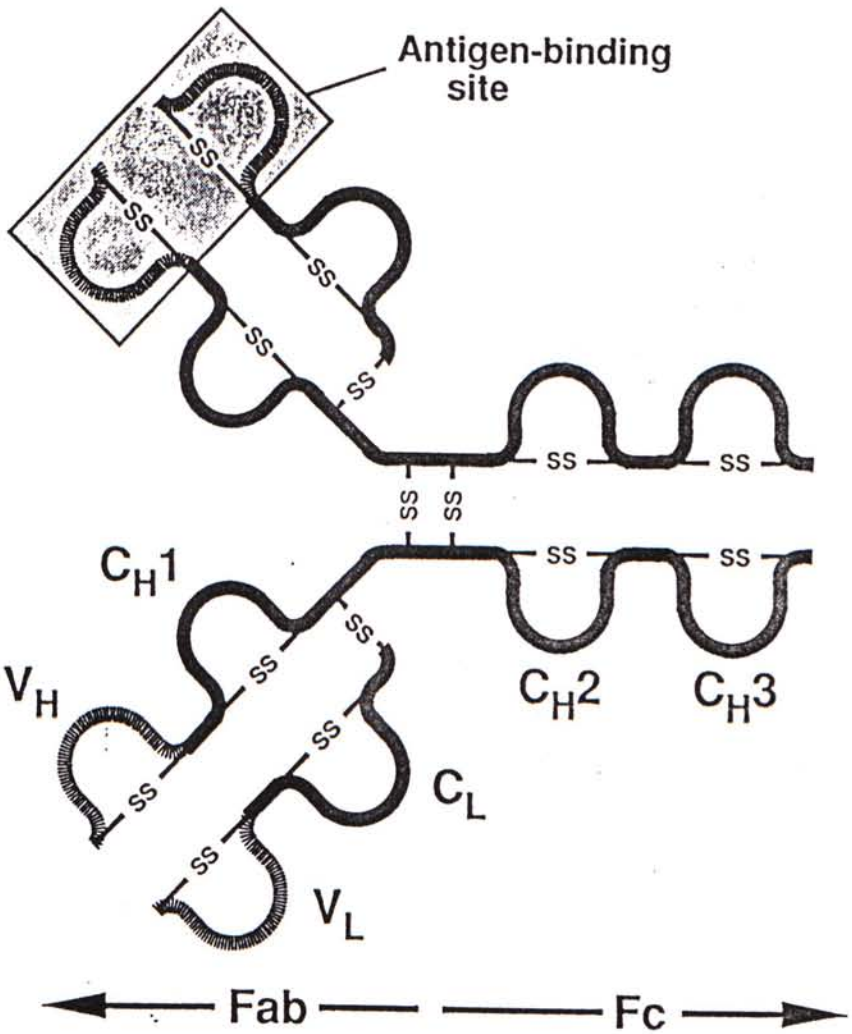
All immunoglobulin molecules consist of a basic unit of two identical heavy (H) and two identical light (L) chains joined by disulfide bonds (Figure 1.1). There are two types of L chains and five classes of H chains. They differ from each other in antigenicity, carbohydrate content and also in size.

There are also expressed several heavy chain subclasses of immunoglobulins. Four subclasses of immunoglobulin G (IgG), namely IgG1, IgG2, IgG3 and IgG4 and two subclasses of immunoglobulin A (IgA), namely IgA1 and IgA2 had been found. The immunoglobulin subclasses have approximately 95 % structural homology with other subclass molecules of the same immunoglobulin type but significantly different from that of the other immunoglobulin isotypes.

1.2 Historical perspective

IgG subclasses were discovered in 1960 [1] but it was not until 1964 [2] that their existence became recognized. Most of the investigations used myeloma proteins due to their monoclonal nature, available with high purity and in high concentrations.

In 1960, Dray [1] immunized rhesus monkey with normal serum γ-globulins (essentially IgG) prepared by cellulose ion-exchange chromatography. The harvested antisera, when used natively and after absorption with different myeloma proteins, were used for immunoelectrophoresis studies. On immunoelectrophoresis, antibodies directed against three different proteins (designated γ-A, γ-B and γ-C) of the γ -globulins were demonstrated. Another group of researchers [2] carried out an investigation similar to that of Dray's. Essentially, they demonstrated three



A simplified model of a human IgG1 immunoglobulin molecule, showing the basic four-chain structure and domains (depicted as *loops*). V = variable region; C = constant region; L = light chain; H = heavy chain. *Thick lines* represent immunoglobulin chains; *thin lines with "SS"* represent disulfide bonds. The Fab (antigen-binding) portion of the molecule is to the left of the two disulfide bonds between heavy chains, in the hinge region of the molecule. It includes the antigen-binding sites formed by the variable regions of light and heavy chains. The Fc portion of the molecule, to the right of the hinge region, gives the molecule certain biologic activities, including Fc receptor binding, placental transfer, serum half-life, and ability to fix complement.

Figure 1.1 An immunoglobulin molecule (From Smith TF. IgG subclass. Adv Pediatr 1992; 39:102)

Another group of researchers [2] carried out an investigation similar to that of Dray's. Essentially, they demonstrated three precipitin arcs (designated as γ^{2a}-, γ^{2b}- and γ^{2c}-globulins) on immunoelectrophoresis of normal human serum with the harvested antiserum. Further investigations were carried out to elucidate the properties of the protein that the antibodies were directed against. Normal human serum and serum from thyroiditis patients were used. They demonstrated that the protein arcs precipitated from the thyroiditis serum specifically bind I^{125} labeled thyroglobulin, binding was not observed in the normal human serum control. This suggested that the antiserum raised from immunising rhesus monkey with γ-globulins produced antibodies directed against antibody molecules in human serum. The location of the antigenic determinant within the γ-globulin molecule was investigation by Ouchterlony analysis after reductive cleavage of the γ-globulins. It was demonstrated that the antibodies produced were directed against the heavy polypeptide chain of the IgG molecules.

Grey *et al.* [3] used a variety of antisera directed against isolated myeloma proteins and successfully distinguished that there are four subgroups of 7S γ-globulins.

These studies [1,2,3] paved the milestones to the discovery and classification of the four IgG subclasses.

1.3 Biochemistry of the IgG subclasses

The four subclasses of IgG in human are designated IgG1, IgG2, IgG3 and IgG4 (Figure 1.2). Their amino acid sequences showed more than 95% homology [4]. Some biological properties of the IgG subclasses are shown in Table 1.1.

The most notable difference between the four subclasses is that IgG3 is heavier than the three other subclasses. The heavier IgG3 is attributed to the presence of significantly longer hinge region as shown in Figure 2. In addition, IgG3 has the

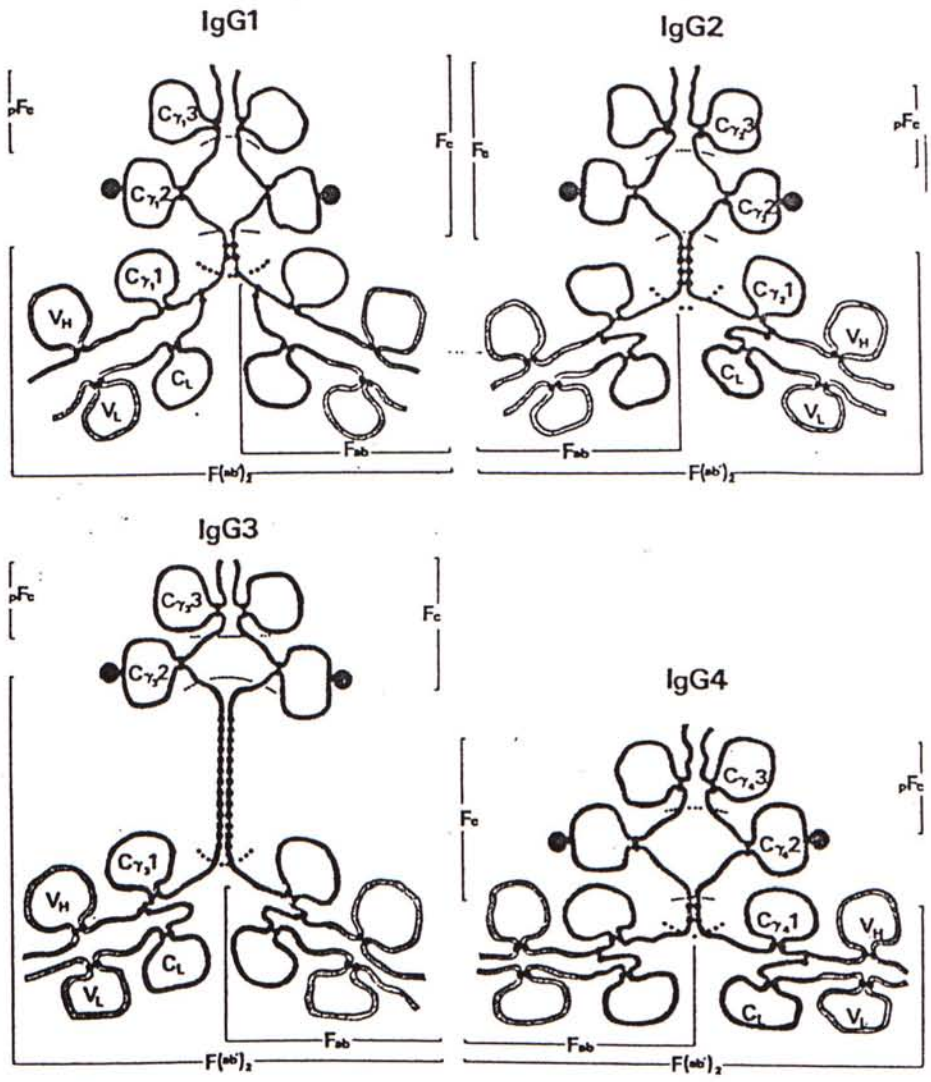


Figure 1.2 Diagrammatic representation of the four IgG subclasses. (From Immunology Today, July 1980)

Subclass	IgG1	IgG2	IgG3	IgG4
Heavy chain	γ1	γ2	γ3	γ4
Half-life (days)	25	23	9	25
Molecular weight	146,000	146,000	165,000	146,000
Complement fixation	++	+	+++	-
Placental transport	+	±	+	+

Table 1.1 Physicochemical and biological properties of IgG subclasses [5]

shortest half-life among the four subclasses and bear the most disulfide bond in the hinge region. IgG1, IgG2 and IgG3 fix complement to varying extent while IgG4 is unable to fix complement. Transfer of IgG subclasses across placenta confers immunity to the neonate immediately after parturition [5].

1.4 IgG subclasses and human diseases

IgG subclasses had been studied in diseases in which there is an involvement of immunological components. Diabetes mellitus, systemic lupus erythrematosis, glomerulonephritis, skin lesions such as pemphigus are only a few to name in which there is an imbalance of IgG subclasses. IgG subclasses in primary antibody deficiency syndrome will be discussed in later section.

1.4.1 Glomerulonephritis

Imai *et al.* [6] studies the role of IgG subclasses in membranoproliferative glomerulonephritis (MPGN), membranous nephropathy (MN) and lupus nephritis (LN) and suggested "IgG subclass determines the phenotype of glomerulonephritis". Difference in serum concentrations as well as glomerular depositions of IgG subclasses varied among the three conditions. Glomerular deposition of IgG1 and IgG2 were found in significant amount in LN. IgG3 and IgG4 were respectively found to be associated with MPGN and MN.

1.4.2 Blistering skin lesions

Bullous pemphigoid (BP), pemphigus vulgaris (PV) and pemphigus foliaceus (PF) are autoimmune diseases resulting in blistering of the skin. These conditions are characterised by circulating IgG autoantibodies against intracellular substance (ICS) and basement membrane of the epidermis.

Jones *et al.* [7] demonstrated a restriction of IgG subclass to intracellular substance within IgG1 and IgG4. Kumar *et al.* [8] has recently demonstrated that IgG basement

membrane autoantibody was primarily of IgG4 subclass and is present in all BP cases. The use of subclass specific antiserum will significantly improve on the diagnostic efficiency of the immunofluorescence test.

1.4.3 Insulin dependent diabetes mellitus (IDDM)

IgG circulating islet cell autoantibodies (ICA) are the serological marker in IDDM. In a study of 51 IDDM patients [9], all of them had IgG1 ICA. A significant difference was observed between patients with IDDM only from those IDDM patients presented with thyro-gastro autoantibodies in that the latter group demonstrated higher concentrations of IgG2, IgG3 and IgG4 ICA. The co-occurrence of IgG ICA of other subclasses in patients with multiple organ autoimmunity suggested a more heterogeneous clonal recruitment pattern.

1.5 Primary antibody deficiencies

A range of primary antibody defects has been recognised and classified as shown in Table 1.2 [10].

1.5.1 CVID

CVID represents a "conglomeration of as yet undifferentiated syndromes" characterised by defective antibody production diagnosed based on exclusion of other defects. Decreased IgG and IgA subclasses followed the order of the heavy chain constant region coding sequences on chromosome 14 with isotypes further downstream being more affected; thus the presentation of variable deficiency of immunoglobulin M (IgM) together with decreased IgA and IgG. [11]

1.5.2 X-linked antibody deficiency

Serum immunoglobulins are low and mature B-cells are absent. The defect is caused by mutations in the Bruton's tyrosine kinase (Btk) gene. Absent of functional Btk leads to failure of B-cell differentiation. [11]

Types of primary antibody deficiencies
• Common variable immune deficiency (CVID) • X linked antibody deficiency • IgG subclass deficiencies • Specific antibody deficiency • Selective IgA deficiency

Table 1.2 Classification of primary antibody deficiencies [10]

1.5.3 IgG subclass deficiency

Criteria for diagnosis is based on a normal IgG level with subnormal or low level of one or more subclasses. Mostly resulted from failure in terminal differentiation of B cells [11]. Deletion of genes cluster coding for the IgG heavy chain had been reported [31,32,33].

1.5.4 Specific antibody deficiencies

The criteria for diagnosis of specific antibody deficiencies include demonstration of an inability to respond to some antigens but normal response to other antigens. Usually total IgG and IgM levels are normal. Association with IgG2 deficiency had been reported. [11].

1.5.5 Selective IgA deficiency

Selective IgA deficiency is prevalent among Caucasians with an occurrence of 1:700. The defect is presumed to be maturational failure of IgA producing lymphocytes. Frequently associated with IgG subclasses deficiencies. [11]

1.6 IgG subclasses deficiency

IgG subclasses deficiency, in its own represent a type of primary antibody immunodeficiency syndrome. However, deficiency of IgG subclasses is invariably associated with other primary antibody deficiency as well as secondary immune deficiency.

In a retrospective study, 4% out of 3005 patients studied were found to suffered from selective IgG1 deficiency [12]. This condition had in the past been confused with CVID. Clinically, 83 percents of these selective IgG1 patients were presented with moderate recurrent infection predominately of the upper respiratory tract.

Patients suffering from ataxia telangiectasis – "a severe hereditary progressive cerebellar ataxia, transmitted as an autosomal recessive trait, and associated with

oculocutaneous telangiectasia, abnormal eye movements, sinopulmonary disease, and immunodeficiency" [13] – were reported to have significantly decreased or even undetectable IgG4 subclass. Out of the 23 (92%) patients who had IgG4 deficiency, only 3 had concurrent IgG2 deficiency [14]. Very low or borderline low IgG2 levels were found in 22 ataxia-telangiectasia patients studied and 19 out of 22 had undetectable IgG4 in their serum [15].

In a study of 73 patients, predominantly children with IgA deficiency [16], 27 patients were found to have IgG subclass deficiencies. Among the 27 patients, 12 of them demonstrated isolated IgG4 deficiency. The remaining 15 had either isolated deficiency of other subclasses or IgG4 in combination with other subclasses deficiency. All patients studied had problems associated with the respiratory tract. Impaired lung function was found to correlate significantly with low IgG2 and IgG3 levels among a group of 29 patients with IgA deficiency [17]. A suggestion that a causal relation exist between low IgG2 or IgG3 levels and impaired lung function was put forward. In fact, the conditions of some patients had benefit from immunoglobulin prophylaxis.

1.7 Clinical Manifestation of IgG subclass deficiency

Schur *et al.* [18] described three patients with the recurrent episodes of otitis media, sinopulmonary infections and progressive pulmonary diseases who were subsequently diagnosed to be deficient in one or more IgG subclasses. These patients present with low IgG1 and combinations of IgG2, IgG3 and IgG4 deficiencies. Their infections responded well to antibiotic therapy. Recurrent infections were prevented upon prophylactic gamma globulins replacement therapy. Recurrent bronchitis, which the investigators [19] defined as "three or more episodes a year, during 2 consecutive years, of bronchopulmonary infection, productive cough

with or without fever and/or diffuse rales by physical examination in the absence of asthma or atopy" is one common clinical presentation of IgG subclass deficiency. Among the 53 children who fulfilled the selection criteria, 30 of them presented with one or more subclasses deficiency. In their study, the incidence of isolated subclass deficiency decreased in the order IgG4 > IgG3 > IgG2.

In another study [20], 7 out of 30 children suffering from recurrent infections had IgG2 deficiency. Only two of these 7 children had isolated IgG2 deficiency. The other 5 had combined deficiency with IgG4 and/or IgA. One of them also suffered from abnormal T cell function. These seven patients suffered from recurrent pneumonia, sinusitis, otitis media, invasive Haemophilus influenzae type b (Hib) infections, as well as severe pneumococcal meningitis.

"The clinical hallmark of IgG subclass deficiencies are RECURRENT respiratory tract infections, including chronic sinusitis, otitis, relapsing pneumonia and bronchiectasis" [21] effectively summarised the presentation of patients suffering from IgG subclass deficiencies. The key word in the statement is the word RECURRENT which illustrate in entire the phenomenon that these patients cannot mount an effective secondary immune response on repeated exposure to the same causative infectious agent.

1.8 Restriction of IgG subclass responses to exogenous antigens

In human, IgG subclass response induced by foreign antigens depends on the nature of the challenging antigens. It had been documented that challenge by polysaccharides and protein antigens elicit different antibody response.

Hammerstrom *et al.* [22] demonstrated that specific IgG subclass antibody to alpha-toxin, a protein toxin of Staphylococcus aureus (S. aureus) was IgG1 with substantial amount in IgG4. Complement fixing antibodies against Streptococcal M-associated

protein (MAP) were found not to be absorbed by protein-A-positive S. aureus [23]. This complement fixing antibody was later found to be of IgG3 subclass and correlated with the properties of this subclass in that it does not interact with protein A.

Umetsu [24] studied the response to Hib capsular polysaccharide antigen in 20 children with selective IgG subclass deficiency and recurrent sinopulmonary infections. IgG2 subclass deficient patients had lower anti-Hib capsular polysaccharide levels while IgG3 deficient patients and control subjects had normal concentrations. To eliminate the possibility of underexposure to Hib polysaccharide (CHO), immunisation of the study subjects with Hib vaccine showed that antibody response to Hib CHO in the IgG2 subclass was significantly lower in IgG2 deficient patients compared with IgG3 deficient patients and normal controls. The observation was in line with that of Siber [25] who demonstrated that antibody response to bacterial polysaccharide antigens resides within the IgG2 subclass.

Specific antibody response towards teichoic acid, a carbohydrate antigen of S. aureus was demonstrated to elicit a response preferentially restricted to IgG2 subclass [22].

Within individual IgGSc antibody response to ovalbumin, avidin and pneumococcal polysaccharide was investigated by Lim and Lau [26] in the light that previous reported IgGSc responses to protein and pneumococcal antigen based on data from different subjects. They demonstrated that with ovalbumin, an association exist between IgG2 and IgG4 and between IgG1 and IgG3. The antibody response to pneumococcal antigen is by and large similar to that investigation by Freijd *et al.* [27] Avidin which is a protein however in terms of its specific IgGSc antibody response, resembles pneumococcal antigen to a larger extent. This suggested that the

carbohydrate moiety of the glycoprotein may be more immunodominant or that the protein fraction is more easily degraded.

1.9 Expression of IgG subclasses

Two hypotheses on B-cell isotype switching had been proposed. A single lineage model and a multilineage model had been proposed. (Figure 1.3)

The single lineage model suggested that a single B cell could express all heavy chain isotypes of immunoglobulins with the same V region by the order of the genes on chromosome 14 [28,29].

In human, IgG2, in which the coding sequence is further down the chromosome, had been demonstrated to secrete preferentially in response to polysaccharide antigen challenge. In addition, exceptions to this IgG2 restriction had been demonstrated in response to Haemophilus influenzae b polysaccharide and pneumococcal capsular polysaccharide. Nahm *et al.* [30] demonstrated that V region of IgG2 antibody against phosphocholine (an immunodominant antigen in the carbohydrate cell wall of Streptococccus pneumoniae) bound phosphocholine (PC) irrespective whether it is conjugated to protein carrier or as carbohydrate antigen. On the contrary, IgG1 antibody will bound only PC antigen conjugated to protein only. Nahm *et al.* suggested that "certain V regions preferentially pair with certain IgG subclasses and supports the multiple B cell lineage model".

1.10 Mechanisms of IgG subclasses deficiency

1.10.1 Gene deletion

The immunoglobulins heavy chain genes are located on chromosome 14. (Figure 1.4) Gene deletion of the immunoglobulin heavy chain cluster had been identified in the genome of a healthy Tunisian Berber and conformed using specific DNA probing

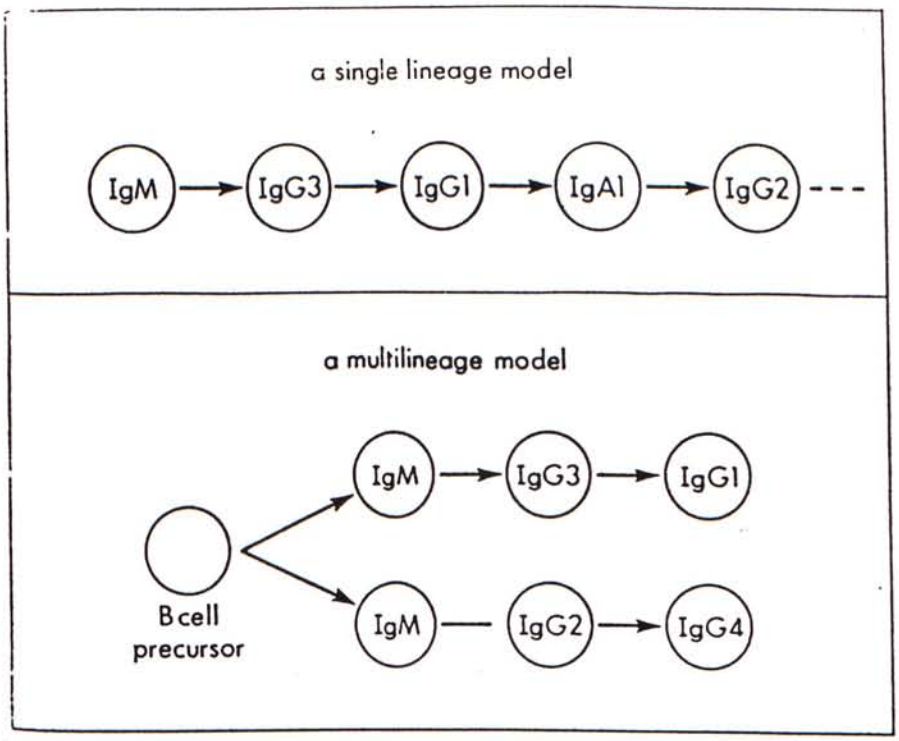
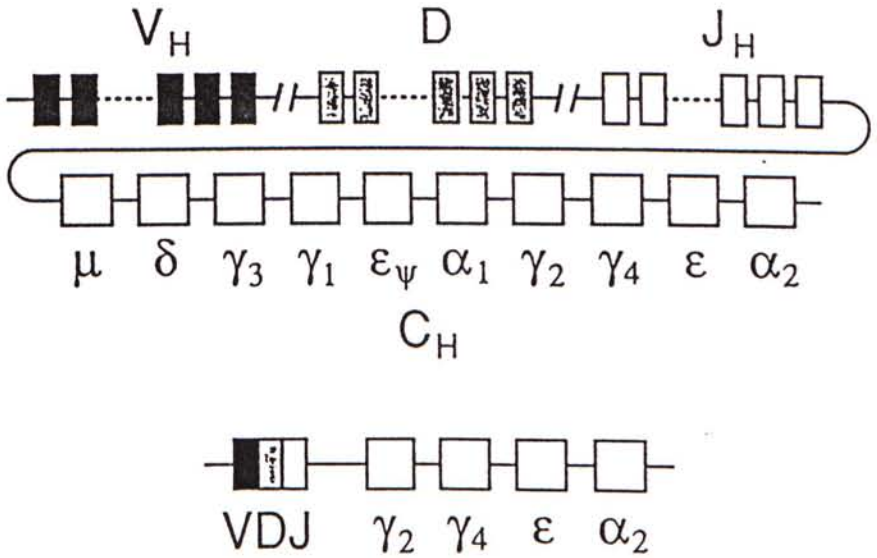


Figure 1.3 Two hypothetical but contrasting models of B cell development (From Nahm MH, Scott MG, Shackleford PG. Expression of human IgG subclasses. Ann Clin Lab Sci 1987; 17:185)



Schematic representation of the human immunoglobulin heavy-chain gene locus. The *upper panel* shows that genes encoding heavy chain variable (V_H) regions, diversity (D_H) regions, and joining (J_H) regions of the antibody molecule are located toward the 5′ end of the chromosome, followed by genes encoding the constant (C_H) domains toward the 3′ end. Each C_H gene encodes three constant domains. Switch regions are located to the 5′ side of each C_H gene. The first DNA rearrangement assembles one V_H gene, one D_H gene, and one J_H gene, to complete the variable portion of the molecule. Association of this VDJ segment with the adjacent Cμ gene promotes transcription of complementary messenger RNA for production of cytoplasmic μ chains. The *lower panel* illustrates a second DNA rearrangement involving switch sites that may result in a more distant C_H gene, such as Cγ2, moving next to the rearranged VDJ segment, as in an IgG2-secreting cell.

Figure 1.4 Schematic representation of the human immunoglobulin heavy-chain gene locus (From Smith TF. IgG subclass. Adv Pediatr 1992; 39:103)

technique [31]. This Tunisian Berber presented with a simultaneous absence of IgG1, IgG2, IgG4 and IgA1 resulted from the multigene deletion.

Chaabani *et al.* [32] identified another deletion in the immunoglobulin heavy chain that includes the alpha-1, gamma-2 and gamma-4 region in one of 32 random Arabo-Berber Tunisian analysed. Other multigene deletions had been reported including an alpha-1, gamma-2, gamma 4 and epsilon found in Sardinian. A similar alpha-1, gamma-2, gamma 4 and epsilon gene deletion was reported in a pair of siblings by Plebani *et al.* [33] in Itlay. The pair of siblings is offspring from a heterozygous father carrying the A1 to E gene deletion and a heterozygous compound mother having deletion in one chromosome but duplication in the other.

In individuals with gene deletions, the overall susceptibility to infections may not be increased. In the earliest study on Tunisian Berber, the subject was healthy [31]. The health status of the subject in Chaabani's [32] study was not mentioned. In the study by Plebani [33], the two siblings were presented with minor upper respiratory infections. On immunisation of these two siblings with tetanus toxoid and polysaccharide antigens, the IgG1 and IgG3 responded to a greater extent when compared with response in all four subclasses in control subjects.

1.10.2 Immune dysregulation

Immune regulatory cells and substances have a role to play in IgG subclass deficiency. B-lymphocytes requires the co-operation of immune regulatory T-lymphocytes and cytokines to function properly. The role of immune regulatory cells and substances on IgG subclasses production were largely obtained through in vitro experiments on cells isolated from subclasses deficient or normal individuals.

1.10.2.1 T-cell receptor defects

A link between T cell receptor (TcR) expression defect and IgG2 deficiency as well as poor antibody response to polysaccharide antigens was investigated by Regueiro *et al.* [34] in a pair of siblings. Both children suffered from TcR immunodeficiency. Antigen challenge by endogenous and exogenous proteins revealed normal immune response. An impaired response was however noted to polysaccharides antigens with IgG2 deficiency. An abnormal T cell receptor was found to be expressed on one of the two siblings studied. The investigators suggested that "the low number of peripheral T lymphocytes that have been found to express low TcR levels in these immunodeficiencies may be operational, and supplying sufficient 'help' for the observed normal antibody responses to all tested protein, but not polysaccharide antigens".

1.10.2.2 Interferon gamma (IFN-γ)

Inoue *et al.* [35] studied on the effect of defective interferon gamma production on IgG2 subclass deficiency. Two pairs of siblings suffering from IgG2 deficiencies were studied. Familial study showed that patients and family members all have sufficient number of immune cells (including) CD3+, CD4+, CD8+and CD14+ as well as CD16+ cells. Response of patients peripheral blood mononuclear cells (PMBC) to phytohaemagglutinin or concanvalin A (Con A) were normal but interferon gamma production was significantly lower than control subjects. Interleukin-2 production by PMBC upon stimulation by Con A was normal in all four patients. When PMBC were stimulated with pokeweed mitogen (PWM) in the absence or presence of interferon gamma, significant elevation in IgG subclasses secretion was observed in the latter. Inoue *et al.* suggested that "the dysfunction in the production of IFN-gamma plays an important role in IgG2 deficiency."

1.10.2.3 Inteleukin-4 (IL-4)

Inteleukins-4 was suggested to regulate IgG subclass in two different ways by Kotowicz *et al.* [36]. IgG subclass production by Epstein Barr virus – T-cell independent polyclonal B cell mitogen -- activated B cells were found to behave differently in the presence of different concentration of IL-4. At a low concentration (5 U/mL), IgG1, IgG2 and IgG3 were produced whereas IgG4 production was induced by IL-4 at a significantly higher concentration (100 U/mL) suggesting the existence of a high- and low- affinity activation pathway for IL-4 mediated B-cell activation.

1.10.2.4 Interleukin-6 (IL-6)

The role of IL-6 in the regulation of IgG subclass production was studied by Kawano *et al.* [37]. In vitro study demonstrated the presence of IL-6 at various stage of the PBMC culture resulted in enhancement of different IgG subclass production. The main action of IL-6 is on the differentiation of committed B cells and triggering of T-helper activity.

1.11 Prevalence of IgG subclass deficiency

In 1988, a study was carried out to study IgG2 deficiency among 8015 blood donors [38]. Among these 8015 healthy blood donors, a total of 312 had IgG2 concentrations of lower than 1.3 g/L (2SD below the mean). This suggested that 4% of healthy individuals who satisfy the criteria of the respective blood transfusion authority for fitness of blood donation had IgG2 deficiency.

Chapel reported for the Consensus Panel for the Diagnosis and Management of Primary Antibody Deficiencies [39] that according to Swedish data on prevalence [40], the British register of primary immune deficiencies included only 40% of affected patients. Furthermore, a diagnostic delay of 2.5 and 5.5 years was noted for

CVID in children and adults respectively suggesting a poor awareness of the condition.

1.12 Reference intervals for IgG subclass

The ontogeny of the four IgG subclasses does not go in parallel. Serum IgG1 and IgG3 levels reach adult concentrations earlier whereas IgG2 and IgG4 levels achieve adult concentrations at around 10 to 12 years old [41,42].

A study was conducted in 1994 by Lau *et al.* [43] to construct percentile range for serum IgG subclasses in healthy Hong Kong Chinese children. The major two subclasses IgG1 and IgG2 were found to increase until the age of 13 and 18 respectively. IgG4 demonstrated the same wide variation in range as had been demonstrated in other studies [42,44] and the phenomenon that a substantial proportion of the studied subjects had undetectable IgG4 [42,45].

1.13 Methods for investigation of IgG subclass deficiency

The methodologies for quantification of IgG subclasses are essentially similar to those available for the quantification of other immune proteins in serum. However, the marked difference in IgG subclass concentrations ranging from the predominant fraction IgG1 to the subclass of lowest concentration, namely IgG4 necessitate careful optimisation of the assays.

Different immunoassay methods are available for the quantification of IgG subclasses in human serum. Methods available ranges from radial-immunodiffusion, enzyme linked immunosorbent assay (ELISA) as well as more automated procedures such as nephelometry and turbidimetry. All these methods require the use of anti-subclass antibody preparations. Monoclonal antisera, and polyclonal antisera render monospecific are available. In addition, the availability of a WHO immunoglobulin Reference Preparation 67/97 [46] served as a consensus to standardisation of assays

using different approach. Assays calibrated against this the reference preparation should exhibit less inter methodology variation in results reported.

1.13.1 Radial-immunodiffusion

Radial immunodiffusion is by far the most popular method used among investigators studying IgG subclasses [24,43]. The principle is by incorporating the subclass specific antisera in agarose and poured onto a thin layer in a plate. Wells are punched on the gel and accurate quantity of sample are added into the wells and allowed to diffuse radially at room temperature. At the equivalent point of antigen-antibody reaction, an intense precipitin ring will be formed.

1.13.2 Enzyme linked immunosorbent assay

This is usually carried out in microtitre plates. The wells of the microtitre plates are coated with subclass specific antibody and unoccupied binding sites are blocked by albumin. Samples are incubated in the wells and IgG subclass will be bound to the solid phase. The next step is to wash away the unbound serum sample in the wells, leaving behind the antibody bound IgG subclasses. A tracer linked second antibody directed against the human IgG subclass molecules but not reactive toward the capturing antibody is then added. In EIA, the most common tracers are enzyme labels such as horseradish peroxide or alkaline phosphatase. The absorbance developed upon addition of substrate is proportional to the concentration of IgG subclass.

1.13.3 Nephelometry/turbidimetry

Turbidimetry and nephelometry quantify IgG subclass concentrations by mean of blocking of incident light and scattering of incident light respectively. Precipitated immune complex in solution will scatter light to varying extent. The higher the concentration of the analyte under consideration, the greater the scattering or the less

the transmitted light. These assays are rarely used as endpoint assays and in automated analysers, usually the parameter recorded is the rate of change of turbidity or light scattering.

1.14 Aim of study

IgG subclass deficiency is a primary antibody deficiency which will be easily missed in the routine investigation of patient when the most common investigation performed is a serum immunoglobulins pattern. In IgG subclass deficiency, the serum total IgG can fall within the normal range. Therefore IgG subclass deficiency can be present despite a normal total IgG level. In view of this situation, the current study is undertaken to:

1. investigate on the performance characteristic of The Binding Site IgG Subclass Beckman Array 360 assays; and
2. investigate on the prevalence of IgG subclass deficiency among patients with recurrent respiratory infections, bronchiectasis and other lung diseases as well as otitis media.

Part 1 of the study is on analytical biochemistry and part II of the study is on the biochemical basis of disease.

CHAPTER 2

MATERIALS AND METHOD I

The Binding Site IgG Subclass Assay

2.1 Materials

2.1.1 IgG subclass assay

The following reagent was obtained from The Binding Site through TWC Research Limited, Hong Kong

- Human IgG subclass Beckman Array kit (Product code: NK001.E)

  The kit contains calibrator set, controls and four bottles of antiserum, one for each subclass.

The following reagents were required for running the IgG subclass assays on Beckman Array 360 and were obtained from Beckman Instruments (H.K.) Limited

- Array buffer (Product code: 663600)
- Array diluent (Product code: 663630)

2.1.2 Evaluation of patients immune status

The following reagents were obtained from The Binding Site through TWC Research Limited, Hong Kong

- Total haemolytic complement kit (Product code: RC001.3)
- Alternative pathway haemolytic complement kit (Product code: RC003.3)

The following reagents were obtained from Beckman Instruments (H.K.) Limited

- IgM assay (Product code: 446610)
- IgG assay (Product code: 446600)
- IgA assay (Product code: 446605)
- C3 assay (Product code: 465330)
- C4 assay (Product code: 465335)
- PFB assay (Product code: 449450)

2.1.3 Apparatus and equipment

- Beckman Array 360 protein system

- The Binding Site jeweler eyepiece

2.2 Evaluation of The Binding Site human IgG subclass assay on Beckman Array 360 protein system

2.2.1 Principle of the Beckman Array Protein System

The Beckman Array 360 protein system employs the principle of rate nephelometry to quantify specific proteins in biological fluids.

When an antigen in serum is mixed with a specific antibody against it, antigen-antibody binding will occur resulting in the formation of immune complex. At optimum antibody and antigen concentration and in the presence of polyethylene glycol, these immune complexes will aggregate to form large lattice and will scatter light. (Figure 2.1) The Array 360 analyser monitors the rate of change of light scattering which is proportional to the amount of analyte present in the biological of interest.

Factors that can be varied to optimise an user–prep chemistry on Array 360 analyser include the gain setting of the analyser, concentration of the antibody and the dilution of the biological sample.

2.2.2 Assay preparation and procedure

Four IgG subclass assays designated G1, G2, G3, G4 were set up in the Array-360 as user-prepared chemistries (UPC). The parameters for calibration run and sample run are shown in Tables 2.1 and 2.2:

From a comparison of Tables 2.1 and 2.2, the difference in UPC setup for calibration and sample (including control) lies in the dilutions used. Samples were assayed at a dilution six times more than that of the calibrators. Literally, this means that the IgG subclass concentration for plotting of the calibration curve is actually six times that was given on the bottle. The Binding Site refers to this value as the 'calibrator

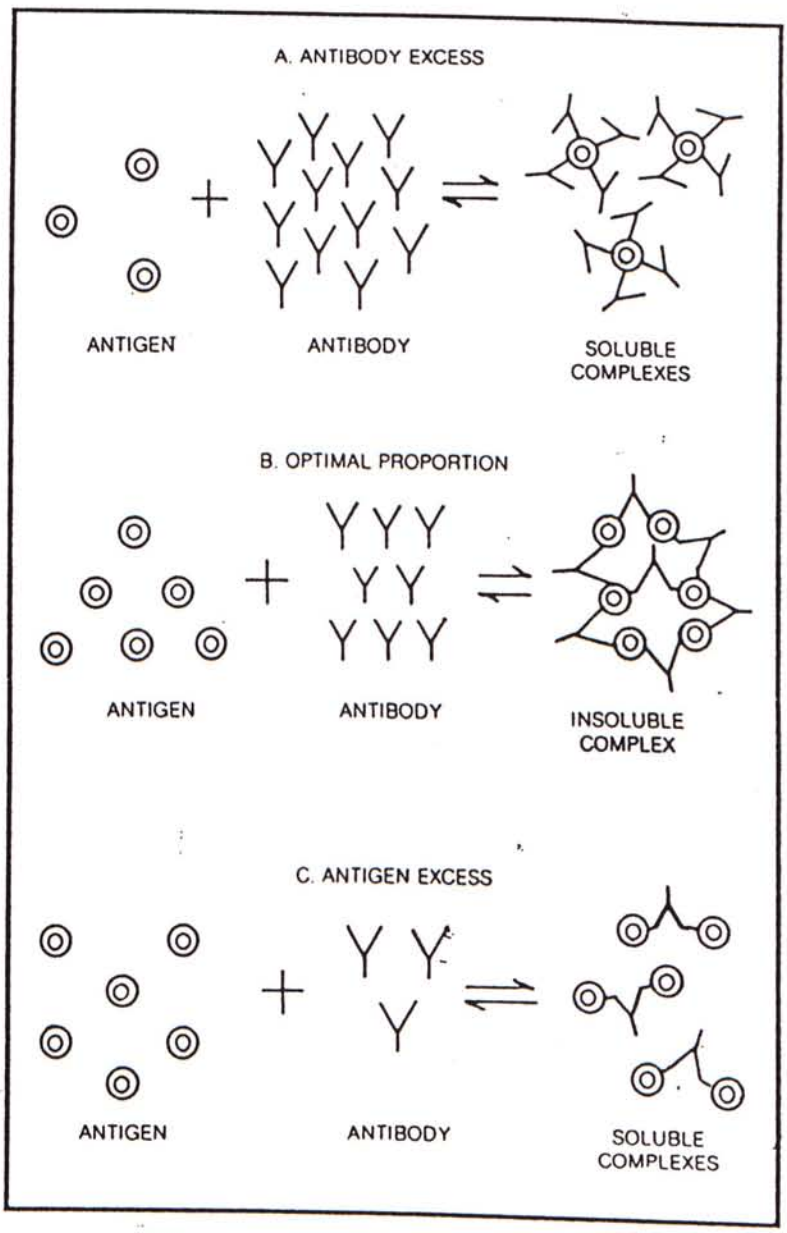


Figure 2.1 Antigen-antibody reaction under varying concentrations of antigen and antibody (From Beckman Array 360 User Manual)

	G1	G2	G3	G4
Gain	22	33	33	33
Dilution	1: 36	1: 6	Neat	Neat
Optics	L	R	R	L

Table 2.1: User-prepared chemistry setup for calibration of IgG subclass assays

	G1	G2	G3	G4
Gain	22	33	33	33
Dilution	1: 216	1: 36	1:6	1:6
Optics	L	R	R	L

Table 2.2: User-prepared chemistry setup for IgG subclass sample assays

representative concentration'. Any sample assayed with the STANDARD sample dilution can be read off directly form a plot of rate against calibrator representative concentration. Any deviation in dilution used for the sample assays will require correction of the results with respect to the standard dilution.

2.2.3 Performance characteristic of the IgG subclasses assay

Investigations on the gain setting, precision, linearity, recovery and interference of the IgG subclass assays were performed.

2.2.3.1 Gain setting

The Binding Site IgG subclass kit used in this study is an optimised assay. No attempt was made in altering the antibody concentration and the sample dilution. The appropriateness of the instrument gain setting was verified by running the same sample using a gain setting 1 step higher and 1 step below that recommended by The Binding Site.

2.2.3.2 Within batch precision

Three levels of pooled human sera were prepared from a single pool. The high pool was prepared by thawing the frozen pool. The top more dilute layer was pipetted off to another pool for preparation of a low pool serum. The original pool was used as the medium pool.

2.2.3.3 Interassay precision

In this study, the low end of the assay range is the area of interest. Three levels of between run precision were studied by using the neat low control, and two other pools prepared by dilution of the low control with calibrator 8 that came with the reagent kit.

2.2.3.4 Linearity of the assay

A pooled human serum sample was assayed for its four IgG subclass levels. The pooled sample was then concentrated to 1/3 its original volume by removing the serum water content by freeze drying technique. The resulting serum was separated into four different portions. Each portion was reconstitute to a volume such that the subclass concentration falls near to the top of the linearity claimed by The Binding Site. These sera were then doubling diluted and assayed to assess the linearity and detection limit of the assay.

2.2.3.5 Interference of the IgG subclass assay

Haemolysis

A whole blood sample was washed ten times with saline to remove the entire plasma protein component. The packed red cells were suspended in limited amount of saline. The haemoglobin content of the saline suspended red cells was measured. The saline suspended red blood cells sample was frozen and thawed twice to prepare the haemolysate for interference study.

A visually clear serum sample was spiked with the haemolysate. This served as the haemolysed sample. Another aliquot of the same serum sample was similarly spiked with reagent grade water. The IgG subclass concentrations of the two serum samples were assayed to assess the interference due to haemolysis

Lipaemia

The approach adopted was similar to that for haemolysis. A human serum with low endogenous triglycerides was spiked with Lipofundin, an IV solution containing glycerol as well as triglycerides. In order to obtain the true concentration of triglycerides spiked, a triglycerides-glycerol blanked assay was set up on the

Beckman Synchron analyser to measure the triglycerides content of the diluted Lipofundin solution.

2.2.3.6 Recovery experiment

Recovery experiment of the IgG subclass assays was conducted on two randomly chosen samples. Aliquots of these two samples were spiked with calibrator 1 and calibrator 5 of the IgG subclass assays calibrator set lot no 25395 using volumes according to Table 2.3

The four recovery pools and the two neat samples were assayed in duplicates. The results obtained were used for recovery calculation.

	Serum X (μL)	Serum Y (μL)	Calibrator 1 (μL)	Calibrator 5 (μL)
Recovery pool X low	180			20
Recovery pool X spiked high	180		20	
Recovery pool Y spiked low		180		20
Recovery pool spiked high		180	20	

Table 2.3: Volume of sample and calibrator for preparation of spiked samples for IgG subclass assays recovery experiment

CHAPTER 3

MATERIALS AND METHOD II

IgG Subclass Deficiency in Hong Kong

3.1 Patients and controls

Fifty-three patients were recruited from Prince of Wales Hospital and Kwong Wah Hospital with recurrent sinopulmonary infections. Twenty-six controls were identified from colleagues and their relatives on the criteria that they did not suffered from recurrent infections, and had no recent infection.

3.2 Blood samples

A 7mL venous blood was collected from each patients and control subjects, and allowed to clot at room temperature for an hour. Serum was separated from the clot by centrifugation at 4°C for 15 minutes at 3000rpm. All samples were stored at –70°C prior to being assayed for the following:

Total IgA

Total IgG

Total IgM

C3

C4

Properdin factor B (PFB)

Total haemolytic activity

Alternative pathway haemolytic activity, and

IgG subclasses

Total IgG, IgA, IgM, C3, C4 and PFB were assayed on the Beckman Array 360 Protein System using reagents manufactured by Beckman Instruments. Calibrations were made against International Federation of Clinical Chemistry (IFCC) standardised value. For IgG subclasses, The Binding Site IgG subclass assays were performed on the analyser as user-prepared chemistries.

Control samples were assayed for total IgG and IgG subclasses. Tests for evaluation of humoral immune status were not performed on control samples.

3.3 Serum total haemolytic complement and alternative pathway haemolytic complement assays.

These were performed on the 53 patient samples using the respective assay kits obtained from The Binding Site

3.3.1 Total haemolytic complement

The principle behind the total haemolytic complement assay is one of radial diffusion. Serum complement components diffused radially into the gel containing antibody sensitised sheep red cells. The antibody, which serves as a heamolysin, fixes complement C1. At low temperature, C1 binds to the haemolysin. On incubation at 37°C, the classical complement pathway was activated resulting in lysis of the sheep red cell. The amount of active components in the serum is proportional to the square of the diameter of the lytic zone. Total haemolytic complement activity is expressed as CH100 units.

3.3.2 Alternative pathway haemolytic complement

Low level hydrolysis of C3 results in the formation of C3b molecules. When serum is added to the alternative pathway heamolytic complement assay gel. At 4°C, C3b from the serum will diffuse radially into the gel and binds to the chicken erythrocytes in the gel allowing the formation of C5 convertase. Upon incubation at 37°C, the alternative pathway of the complement cascade will be activated leading to lysis of the chicken erythrocytes. The combined complement activity through the alternative pathway in the serum sample is proportional to the square of the diameter of the lytic zone. Alternative pathway complement lytic activity is expressed as % of normal.

These assays are supplementary to direct measurement of C3, C4 and PFB in a way that a normal level of these three measured parameters did not preclude deficiency of other complement components. The measurement of haemolytic activity required in the serum sufficient concentrations of all the components of the respective cascades.

3.4 Statistical analysis

Statistical analysis of data obtained on IgG subclass deficiency study was performed with Statistica for Windows version 4.2. Since patients and control subjects have been recruited at random, the non-parametric Mann Whitney U test was selected for analysis of data.

CHAPTER 4

RESULTS I: Evaluation of The Binding Site IgG Subclass Array Kit

4.1 Gain setting

The effect of varying the gain setting on the rate signal obtained for the four IgG subclass assays are shown on Table 4.1. The gain settings as recommended by The Binding Site were found to be appropriate for the antibody concentration used in the Array 360 kit.

4.2 Within batch precision

Within batch precision data are shown on Table 4.2. The coefficient of variation varied from the lowest of 0.6% to the highest of 1.9%. All these CV values were comparable if not better than those claimed by The Binding Site.

4.3 Inter-assay precision

Inter-assay precision data are shown on Table 4.3. The coefficient of variation varied from the lowest of 1.4 % to the highest of 4.0%. On average, the CV values were comparable with that claimed by The Binding Site.

4.4 Linearity and lowest limit of detection

Linearity of the four subclasses assays were assessed by assaying doubling dilutions of the concentrated sample as described in section 2.2.3.4. Graphical representations of the linearity experiment results are shown on Figures 4.1-4.4. The lowest limit of detection were found to be 1494mg/L, 318mg/L, 108mg/L and 132mg/L for IgG1, IgG2, IgG3 and IgG4 respectively when assayed using the standard dilutions..

4.5 Interference experiments

The effects of haemolysis and lipaemia on the IgG subclass assays were carried out as described in section 2.2.3.5. Results were tabulated on Tables 4.4 and 4.5.

4.6 Recovery experiment

Recovery experiments were carried out as described in section 2.2.3.6. The results are shown on Table 4.6. The range of recovery ranges from 94% to 114.6%.

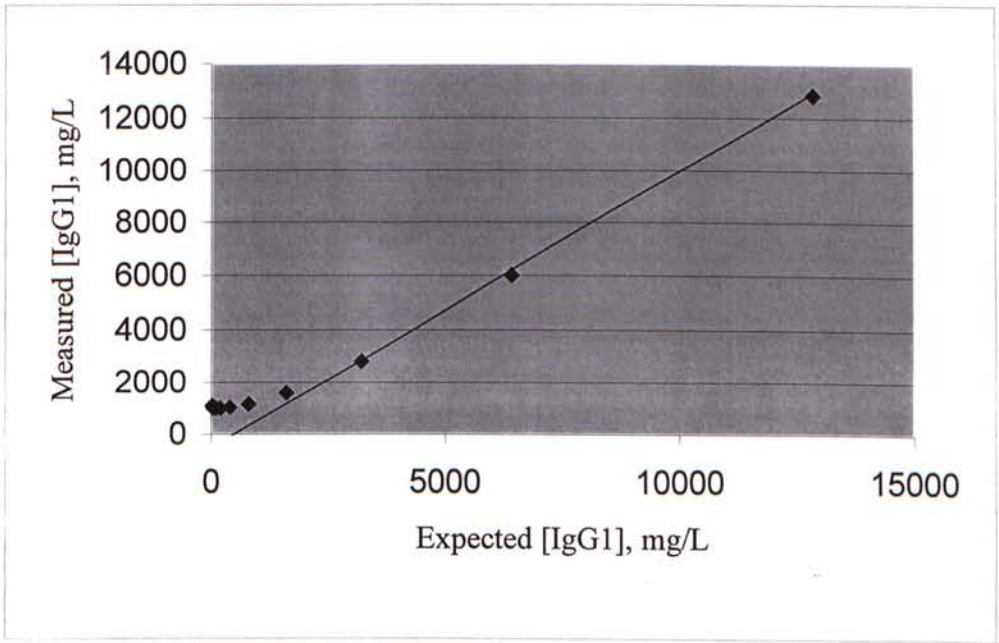


Figure 4.1: IgG1 Assay Linearity Plot

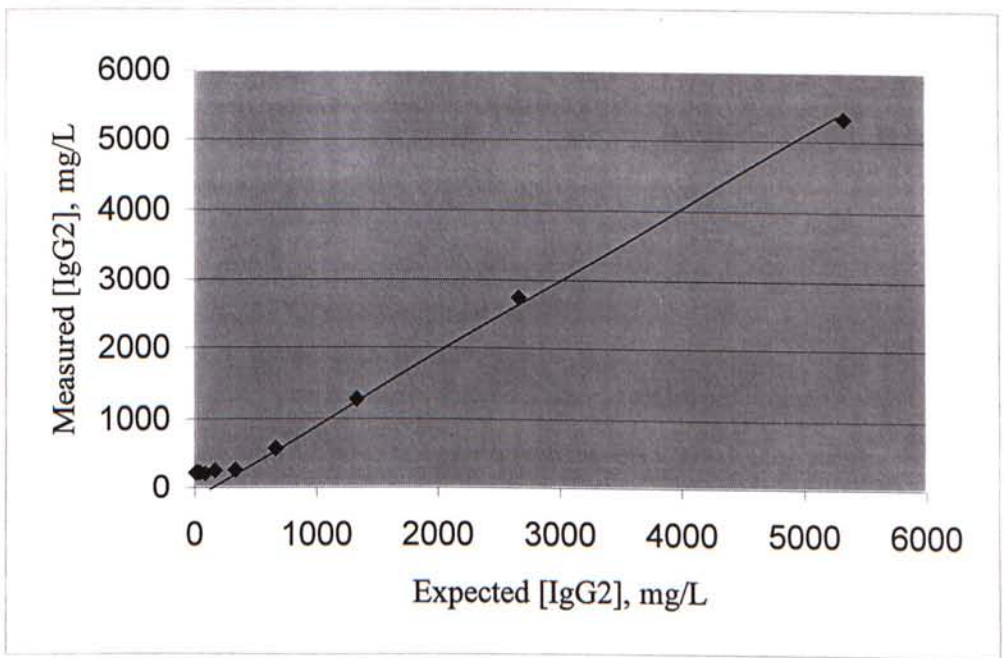


Figure 4.2: IgG2 Assay Linearity Plot

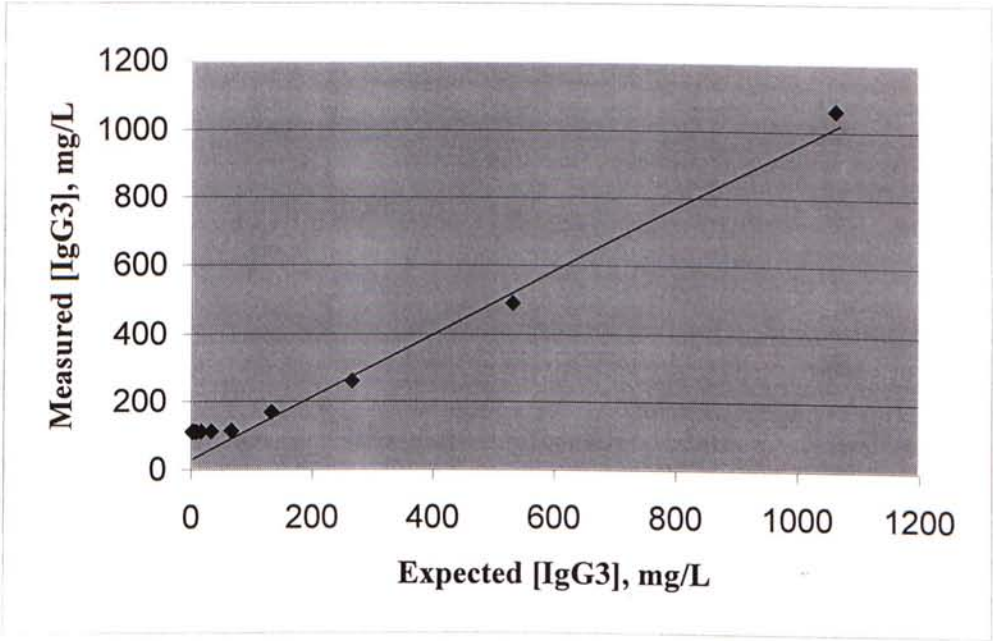


Figure 4.3: IgG3 Assay Linearity Plot

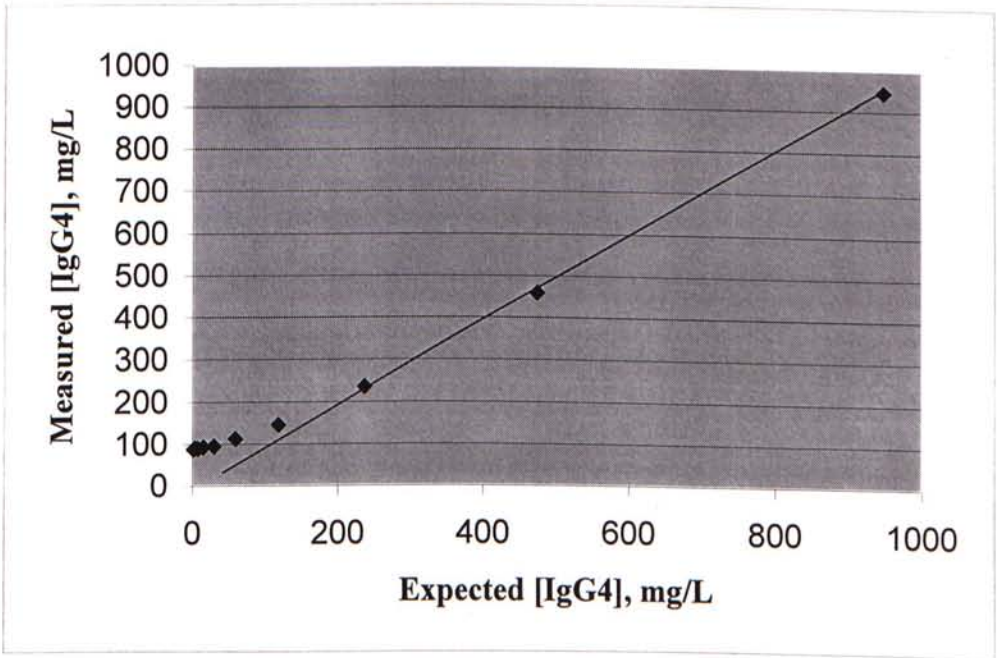


Figure 4.4: IgG4 Assay Linearity Plot

	IgG1	IgG2	IgG3	IgG4
Gain setting	22	11	11	11
Rate unit	439	463	463	254
Gain setting	33	22	22	22
Rate unit	1680	1950	1570	944
Gain setting	44	33	33	33
Rate unit	ES	ES	ES	ES

ES = excess scatter

Table 4.1: Variation in rate signal with change in gain setting for IgG subclass assays

		IgG1	IgG2	IgG3	IgG4
Low	Mean	1231	3255	138	102
	SD	7	46	1	1
	CV (%)	0.6	1.4	0.7	1.0
Intermediate	Mean	3277	5302	371	287
	SD	48	64	6	4
	CV (%)	1.4	1.2	1.5	1.6
High	Mean	6050	6729	522	412
	SD	113	79	7	3
	CV (%)	1.9	1.2	1.3	0.8

All N = 20

Table 4.2: Within batch precision at three levels of IgG subclass concentrations

		IgG1	IgG2	IgG3	IgG4
Low	Mean	1400	709	148	133
	SD	30	23	6	3
	CV (%)	2.1	3.3	4.0	2.5
Intermediate	Mean	1989	1292	193	180
	SD	27	51	4	5
	CV (%)	1.4	4.0	1.8	2.9
High	Mean	3914	2747	363	310
	SD	81	118	7	12
	CV (%)	2.1	4.3	2.0	3.9

N = 10 for IgG1, IgG3 and IgG4

N = 7 for IgG2

Table 4.3: Inter-assay precision at three levels of IgG subclass concentrations

	IgG1	IgG2	IgG3	IgG4
Original	1137	718	85	184
[Hb] 500 mg/dL	1151	731	81	178
% interference	1	2	-5	-4

Table 4.4: Effect of haemolysis on IgG subclass assays.

	IgG1	IgG2	IgG3	IgG4
Original	1284	1049	194	80
Triglycerides (200mg/dL)	1305	1031	160	50
% negative interference	-2	2	17	38
Original	1277	1036	190	72
Triglycerides (400 mg/dL)	1256	1001	138	UR
% negative interference	2	3	27	100

UR = unstable reaction

Table 4.5: Effect of lipaemia on IgG subclass assays.

	IgG1	IgG2	IgG3	IgG4
Recovered	7727	3213	1396	271
Expected	8033	3340	1390	273
% recovery	96.2	96.2	100.4	99.5
Recovered	8022	3966	1402	291
Expected	8266	3461	1412	287
% recovery	97.0	114.6	99.2	101.4
Recovered	2610	2051	298	171
Expected	2614	2076	302	169
% recovery	99.8	98.8	98.5	101.2
Recovered	2776	2065	323	184
Expected	2847	2196	324	183
% recovery	97.5	94.0	99.4	100.6

Table 4.6: Results for recovery experiment of IgG subclass assays

CHAPTER 5

RESULTS II: IgG Subclass Deficiency in Hong Kong

5.1 IgG subclass concentrations and humoral immune status evaluation results of patients and control subjects

All the test results are tabulated on Table 5.1.

5.2 Statistical tests

Results of Mann Whitney U test on IgG subclass concentrations of adult patient and control groups are shown on Table 5.2 and Whisker plots (Figures 5.1-5.4)

Result of Mann Whitney U test on IgG2 concentration of adult patients suffering from bronchiectasis and control group is shown on Table 5.3.

Sample no	IgG g/L	IgA g/L	IgM g/L	PFB mg/dL	C3 mg/dL	C4 mg/dL	IgG1 mg/L	IgG2 mg/L	IgG3 mg/L	IgG4 mg/L	Sum IgGSc g/L	Sex	Age	Diagnosis
1	20.7	4.8	1.5	49	268	58	13349	7537	1824	555	23.3	M	35	bronchiectasis
2	12.5	4.0	0.7	40	97	40	9086	3036	566	710	13.4	F	71	MRSA
3	16.8	2.4	3.3	41	90	19	12459	4309	1169	743	18.7	F	60	type II respiratory failure
4	17.3	5.2	1.8	38	93	21	8826	7702	995	656	18.2	F	40	haemoptysis
5	21.7	2.9	1.4	24	90	19	12830	7726	3275	164	24.0	F	35	bronchiectasis
6	16.0	1.9	1.4	26	107	23	10124	5628	1292	737	17.8	F	38	bronchiectasis
7	9.7	2.5	3.2	46	129	31	7529	1724	903	269	10.4	M	3	bronchitis obliterans
8	17.0	3.6	2.7	40	147	34	8900	6854	2089	650	18.5	F	14	bronchiectasis
9	17.8	11.6	0.6	27	75	19	11014	6288	907	1717	19.9	F	70	RA, bronchiectasis
10	11.6	3.3	0.9	33	72	21	8048	3107	473	1652	13.3	M	80	recurrent MRSA infection ? immunodeficiency
11	10.6	6.1	1.9	23	102	24	8159	2122	768	261	11.3	M	9	bronchitis obliterans bronchiectasis
12	8.2	1.2	0.9	35	134	24	5601	2086	375	231	8.3	M	5	bronchiectasis
13	14.1	4.5	1.6	33	103	27	9271	4709	1439	251	15.7	F	74	asthma
14	10.4	3.1	0.7	52	117	31	6861	3460	749	451	11.5	M	75	bronchiectasis
15	8.2	3.5	2.4	26	91	19	5193	3013	792	152	9.1	M	9	bronchiectasis 15 = 24
16	19.2	4.1	1.4	40	129	44	14239	4309	2101	1257	21.9	M	7	bronchiectasis tb
17	21.9	3.9	1.9	55	143	43	18131	3531	1268	537	23.5	M	49	haemoptysis
18	9.1	1.4	1.3	48	127	32	7232	1660	717	721	10.3	F	4	recurrent pneumonia bronchiectasis
19	11.4	1.5	3.1	34	137	29	9086	2343	472	797	12.7	F	4	bronchitis obliterans
20	18.3	4.1	1.3	34	113	35	11718	6194	3842	421	18.3	F	73	bronchiectasis
21	12.3	3.2	1.3	33	102	24	8085	4120	895	1100	14.2	F	69	bronchiectasis
22	12.9	4.1	1.4	32	97	22	6713	6265	856	894	14.7	M	47	bronchiectasis
23	11.0	2.2	0.9	39	114	33	7566	3225	1828	210	12.8	M	82	bronchiectasis
24	8.4	3.1	1.9	43	158	27	5490	2801	939	204	9.4	M	9	bronchitis obliterans bronchiectasis
25	16.6	6.6	1.1	39	113	30	9753	6500	1954	1094	19.3	F	67	bronchiectasis
26	9.3	1.4	1.9	64	148	43	6342	2683	1474	209	10.7	F	3	asthma
27	11.8	3.8	0.6	32	98	30	8752	2942	1014	295	13.0	F	66	bronchiectasis
28	14.4	2.8	0.6	38	112	29	9642	5440	1133	307	16.5	F	57	bronchiectasis
30	16.9	9.1	0.8	53	125	28	13275	3248	2990	202	19.7	M	71	haemoptysis
31	19.3	37.4	0.9	43	109	34	14610	4568	1724	266	21.2	F	74	bronchiectasis
32	10.5	2.8	0.4	33	132	34	6305	4144	1090	263	11.8	F	73	bronchiectasis
33	21.7	3.4	1.7	39	114	30	17686	3343	1248	525	22.8	M	49	bronchiectasis
34	9.0	0.7	1.1	65	183	44	7937	760	1006	206	9.9	M	10m	acute bronchiolitis
35	23.5	3.2	2.1	42	73	18	19206	3767	2101	158	25.2	M	1	bronchitis obliterans
36	13.0	4.6	1.8	26	114	34	10124	3437	1006	169	14.7	F	13	bronchitis obliterans bronchiectasis
37	13.6	2.1	2.2	29	97	27	8715	5275	1225	140	15.4	F	35	bronchiectasis
38	11.4	1.9	0.7	45	102	26	6824	4756	290	613	12.5	M	57	bronchiectasis
39	15.6	3.4	2.4	36	105	28	11384	4780	1117	600	17.9	M	68	bronchiectasis
40	11.9	1.7	0.7	37	119	22	9642	2522	935	219	13.3	F	5	bronchiectasis recurrent chest infection
41	13.8	1.9	1.3	21	101	21	9864	3767	1300	558	15.5	M	9	bronchitis obliterans
42	12.3	3.5	1.1	31	113	35	8530	3625	1724	306	14.2	F	60	bronchiectasis
43	22.2	4.7	1.4	40	119	32	14721	7325	2046	549	24.6	M	35	bronchiectasis 1=43

Figure 5.1 IgG subclass results and humoral immune status evaluation results. (Sample no with prefix C are controls)

Sample no	IgG g/L	IgA g/L	IgM g/L	PFB mg/dL	C3 mg/dL	C4 mg/dL	IgG1 mg/L	IgG2 mg/L	IgG3 mg/L	IgG4 mg/L	Sum IgGSc g/L	Sex	Age	Diagnosis
44	10.3	0.6	1.3	43	134	29	7825	2145	1744	242	12.0	F	6m	? immunodeficiency
45	8.7	2.5	1.0	31	101	29	5156	3578	594	310	9.6	M	62	bronchiectasis
46	16.8	2.1	1.7	44	123	36	11570	5393	1225	469	18.7	F	37	bronchiectasis
47	4.6	<0.07	0.1	41	115	26	2420	2042	169	114	4.7	M	17	hypogammaglobulinaemia, no active infection
48	59.3	18.4	1.4	18	64	15	33442	6830	2070	13093	55.4	M	62	tb
49	12.9	0.7	1.0	50	142	32	10272	3272	876	1224	15.6	M	1	recurrent chest infection, bronchiectasis
50	18.2	<0.07	1.1	31	111	20	16167	1728	641	351	18.9	F	20	IgA deficiency IgG2 deficiency bronchiectasis
51	12.2	2.2	1.7	40	117	31	8567	3743	967	119	13.4	M	11	Kartogenie syndrome
52	14.5	2.2	1.8	32	150	23	8493	5558	1463	226	15.7	F	8	bronchiectasis biliary dyskinesia
53	11.0	1.4	1.5	23	97	16	7603	3060	733	349	11.7	F	8	bronchiectasis
54	16.6	4.4	0.9	32	132	43	11903	7089	955	278	20.2	M	14	??? syndrome
C1							9816	2479	657	504		F	2.0	
C2							8740	2903	700	1146		F	12.0	
C3							7524	5550	720	345		F	17.0	
C4							5934	7731	419	255		F	30.0	
C5							6823	5059	1205	834		F	35.0	
C6							5139	5796	774	783		F	35.0	
C7							6121	7209	716	407		F	39.0	
C8							9254	5734	1276	254		F	40.0	
C9							7805	4598	1154	455		F	41.0	
C10							9816	5734	1456	1085		F	42.0	
C11							5391	5765	172	238		F	45.0	
C12							7150	6656	1135	424		F	45.0	
C13							9301	8406	1142	408		F	47.0	
C14							6682	5919	931	681		F	57.0	
C15							7805	5089	931	500		F	70.0	
C16							7056	5243	821	875		M	29.0	
C17							7851	4322	290	743		M	29.0	
C18							5794	5550	242	251		M	35.0	
C19							6074	5550	774	957		M	40.0	
C20							6682	4137	492	589		M	44.0	
C21							7477	2986	633	202		M	46.0	
C22							6963	3922	504	491		M	51.0	
C23							6355	4936	528	310		M	51.0	
C24							5293	4844	704	1110		M	52.0	
C25							9301	5734	1037	1146		M	53.0	
C26							6074	4844	1479	487		M	58.0	

Figure 5.1 (cont'd) IgG subclass results and humoral immune status evaluation results. (Sample no with preficx C are controls)

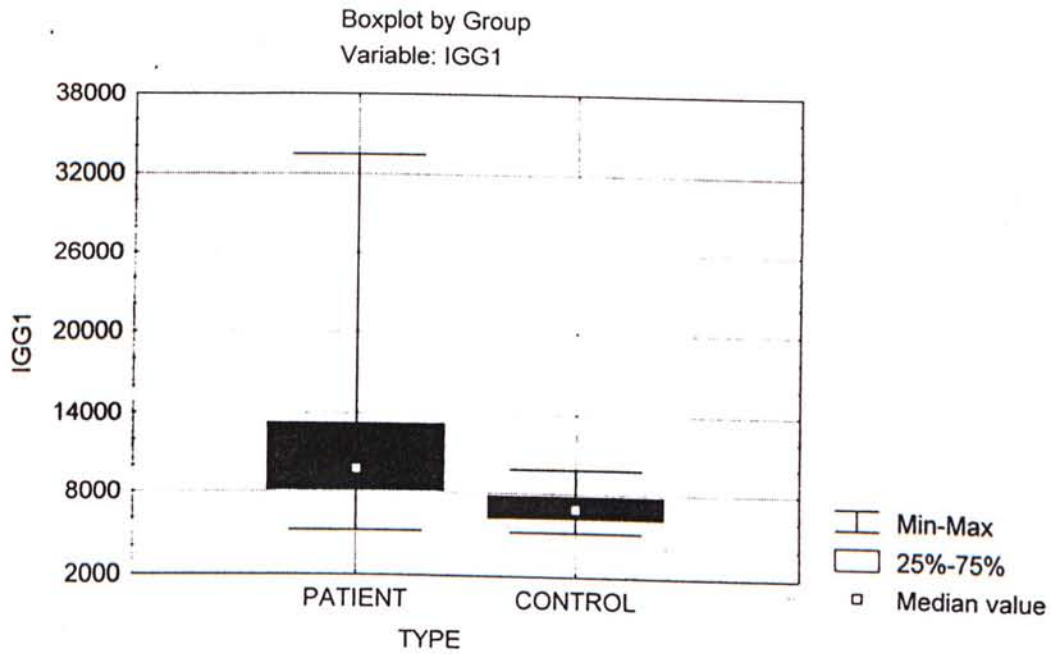


Figure 5.1: IgG1: Patients and Controls Distribution

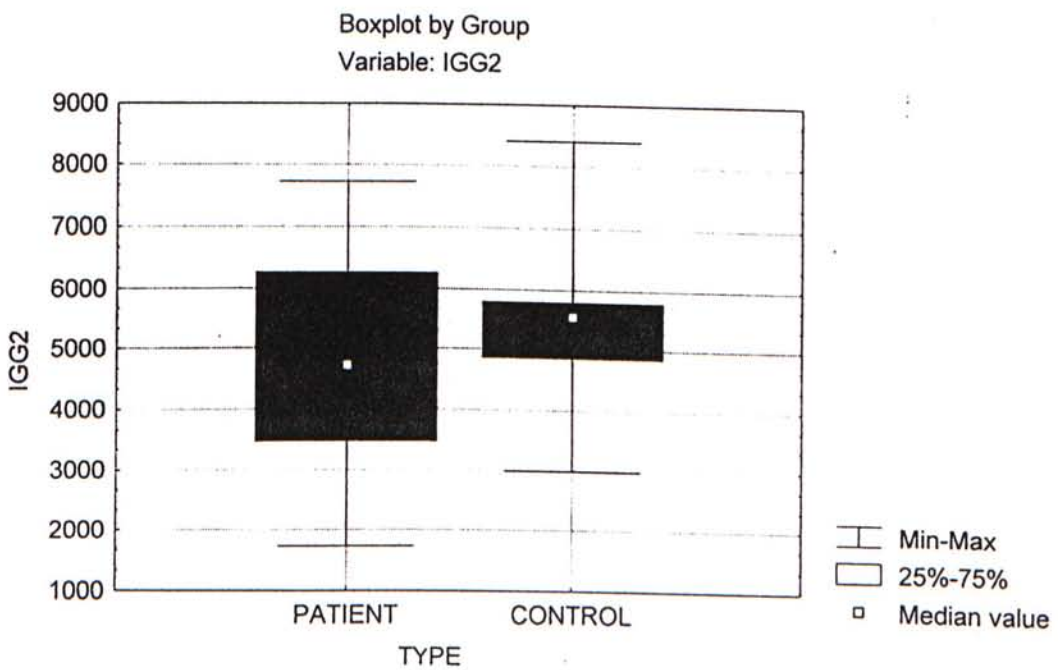


Figure 5.2: IgG2: Patients and Controls Distribution

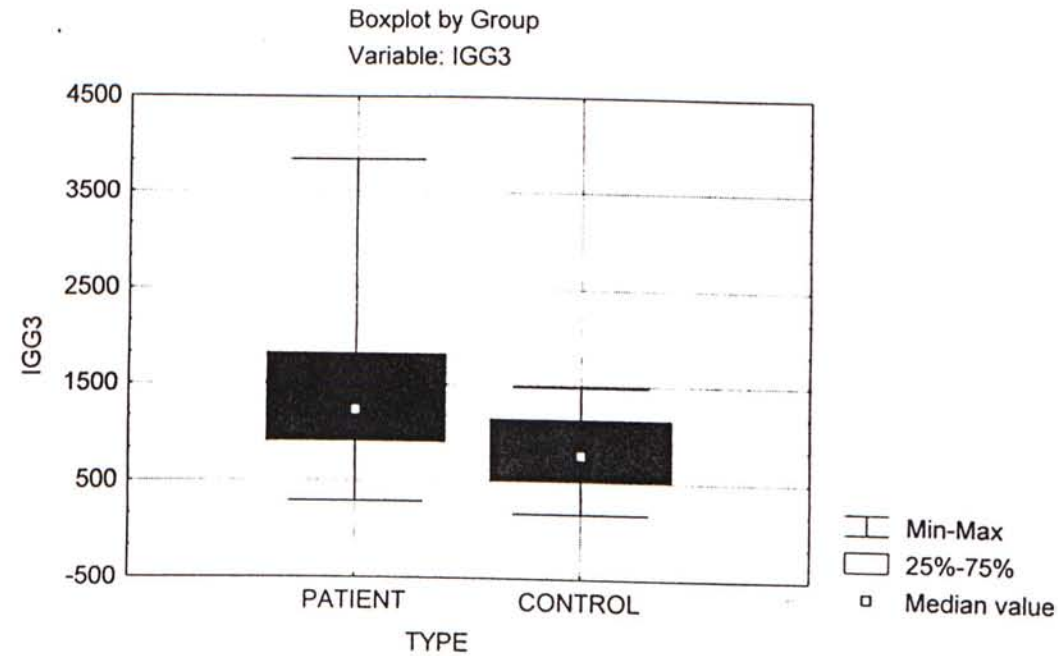


Figure 5.3: IgG3: Patients and Controls Distribution

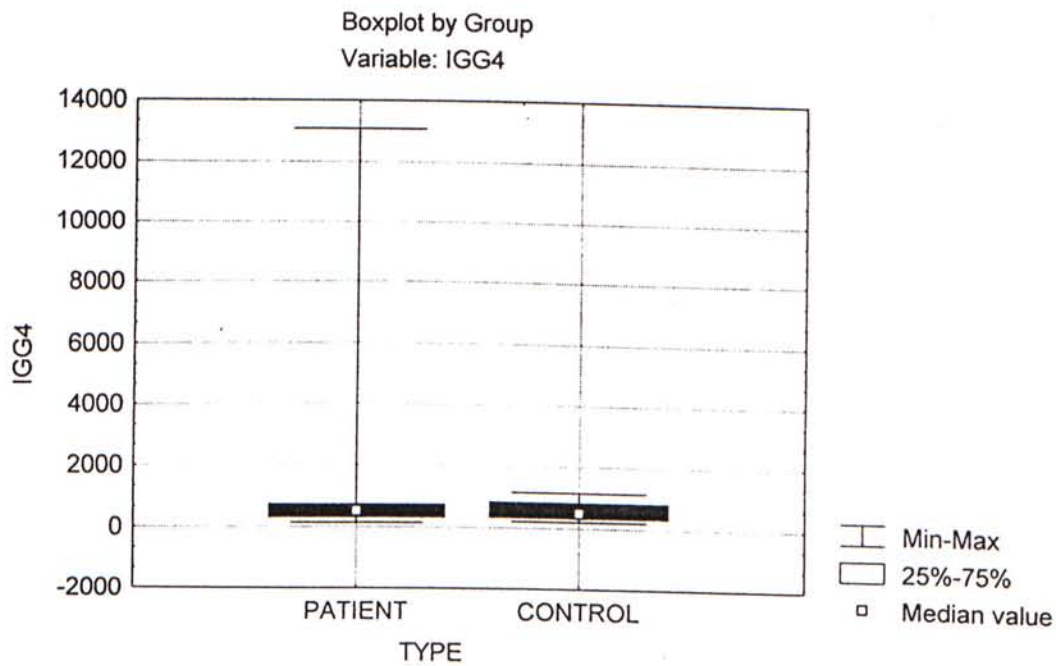


Figure 5.4: IgG4: Patients and Controls Distribution

Mann-Whitney U Test
By variable A
Group 1: 100-PATIENT Group 2: 101-CONTROL

	Rank Sum PATIENT	Rank Sum CONTROL	U	Z	p-level	Z adjusted	p-level	Valid N PATIENT	Valid N CONTROL
İgG1	1098	387	111	-4.294534	1.76E-05	-4.294861	1.75E-05	31	23
IgG2	758	727	262	-1.653089	0.098322	-1.653278	0.098284	31	23
IgG3	1023	462	186	-2.982558	0.002861	-2.982728	0.002859	31	23
IgG4	833	652	337	-0.341114	0.73302	-0.341114	0.73302	31	23

Table 5.2 Result of Mann Whitney U Test: comparison of IgG subclass concentrations between patient and control group

Mann-Whitney U Test
By variable A
Group 1: 100-PATIENT Group 2: 101-CONTROL

	Rank Sum PATIENT	Rank Sum CONTROL	U	Z	p-level	Z adjusted	p-level	Valid N PATIENT	Valid N CONTROL
IgG2	510	618	210	-1.404573	0.160158	-1.404817	0.160085	24	23

Table 5.3 Result of Mann Whitney U Test: comparison of IgG2 sublcass concentration between bronchiectastic patients and controls

CHAPTER 6

DISCUSSION I: The Binding Site IgG Subclass Array Kit

6.1 IgG subclass assays

The IgG subclass assay is based on the principle of rate nephelometry. Antigens and antibodies are mixed together in the presence of ethylene glycol, a reagent, which enhance the lattice formation of the immune complex. The maximum rate of change of the scattering signal is proportional to the antigen concentration.

6.2 Within batch and inter-assay precision

The within batch and inter-assay precisions as shown on Tables 4.2 and 4.3 are comparable with that given in the package insert. The CV values ranges from the lowest of 0.6% to the highest of 1.9% for within batch precision and from 1.4% to 4.3% for inter-assay precision. All within batch and inter-assay CV are comparable with that quoted by The Binding Site.

6.3 Lowest limit of detection

The lowest limit of detection is defined as the point in which the curve constructed by doubling dilutions of a high sample deviates from linearity. In order to eliminate the source of human error in fitting the curve for linearity, the lowest limit of detection was taken as the intersection point of line of best fit connecting the top four samples for linearity assay with the best fit lines connecting the lower six samples. The results so obtained are comparable with that claimed by The Binding Site.

The effective lowest limit of detection for IgG3 and IgG4 are the same as that determined experimentally. For IgG1 and IgG2, the effective lowest limit of detection can be lowered to a level 36 times and 6 times less than that determined experimentally. The rationale behind this is that for these two assays, samples of very low values can be assayed using a sample 36 times more concentrated and six time more concentrated than the standard dilutions of 1:216 and 1:36 respectively for IgG1 and IgG2 thereby lowering the limit of detection. In the IgG subclass assays,

the lowest possible sample dilution that can be used is 1:6; neat serum sample is never used owing to the fact that if the matrix of a neat sample creates a high lower signal to noise ratio and hence the assay will lost sensitivity.

6.4 Interference

Interference experiments were carried out as described in section 2.2.3.5

Haemolysed sample at a haemoglobin concentration of 500 mg/dL did not significantly affect the results of IgG1 and IgG2 assays. For both IgG3 and IgG4, which were assayed at a dilution of 1:6, a slight negative interference was observed. Lipaemia, arising as a result of light scattering molecules in serum, will definitely affect an assay using nephelometry in which the scattering of light is used as the parameter of quantification. As expected, degree of interference varied with the dilution of the serum sample used. At a dilution of 1:6 for IgG3 and IgG4, significant negative interference on the result was observed at a triglycerides concentration of 200mg/dL. At 400mg/dL, the assay for IgG4 returned as unstable reaction while IgG3 suffered a negative interference of 27%. On the other hands, lipaemia at 400 mg/dL did not significantly affect the performance of IgG1 and IgG2 at the standard dilution used for analysing these two subclasses, i.e. at 1:216 and 1:36. However, it can be projected from the performance of IgG3 and IgG4 that if the IgG1 and IgG2 are to be assayed at smaller dilutions as in the case of sample with low subclass values, the same phenomenon of interference would be observed.

6.5 Recovery of IgG

As shown on table, the recovery ranges s from 96% to 114.6% and unfortunately, the lowest and highest recovery were with IgG2 subclass assay at a high level. There is no logical explanation for this observation and warrants further investigation.

6.6 Overall performance of the nephelometric assay

The IgG subclass assay on the Array 360 protein system can be easily setup. Familiarisation takes less than half an hour on an individual already trained to operate on the analyser. Array 360 has an additional capability of storing not only test combination but also sample dilutions in its panel definition. This feature is made use of in defining panels for calibration.

The user prepared chemistries used dedicated optic on the Array 360 analyser thus allowing easy trouble shooting of an assay when the performance was poor. The nephelometric assay for IgG subclass was fast to perform. Results were generated at less than 3 minutes per sample of 4 subclasses by using the left and right optic simultaneously. Together with the enhanced reproducibility of an automated assay, the IgG subclass should be a method of choice for provision of service.

CHAPTER 7

DISCUSSION II: IgG Subclass Deficiency in Hong Kong

7.1 IgG subclass deficiency in adults

As described in section 3.1, thirty-one specimens from 29 adult patients (2 patients contributed two pair of samples in two different episodes) were compared with 23 adult controls. Statistical test by Mann-Whitney U test detected no significance difference between the patient group and the control group for IgG2 and IgG4 concentrations. IgG1 and IgG3 displayed significant differences between the patient group and control group. However, the differences between the two groups were such that the patient group has higher IgG1 and IgG3 concentrations than the control group as shown by Figures 5.1 and 5.3.

In the context of IgG deficiency, the 29 patients studied were not deficient statistically. The patient group included patients with recurrent infections. The significantly higher concentrations of IgG1 and IgG3 could be explained by the fact that these patients are not IgG subclass deficient. They can mount an immune response to infections and their higher concentrations of IgG1 and IgG4 subclasses would not be surprising.

In this pilot study, resources have not been available for setting up of a reference interval for the four IgG subclasses. This is especially true for the pediatric reference intervals, which are divided into groups of 2 to 3 years' intervals. Though Mann Whitney U test revealed no difference in the between the patient group and control group, an attempt was made to study individual patients with respect to the reference intervals quoted by The Binding Site which are based on populations in the Birmingham area, United Kingdom. The Binding Site reference intervals quoted include the mean and the 95 percentile range. In the comparisons that follow, individual IgG subclass concentrations are examined if it falls below the mean or 2 times the lower 2.5 percentile value whichever the lower.

A review of the IgG1 and IgG4 results of the studied adult patient group revealed no single results for these two subclasses fall lower than the criteria. For the control subjects, two females displayed IgG1 concentrations 5139 and 5391 mg/L respectively. These values fall short of the reference point by less than 15%. The mean of the IgG subclass The Binding Site IgG1 reference intervals demonstrated a bias toward the lower percentiles. It would therefore be reasonable to assume that these two results could well not be clinically significant.

IgG2 concentrations of the entire control group are higher than the reference point for further examination. Two female patients however have concentrations below the reference point, one of them by 10 mg/L and were considered unremarkable. The second result was from a patient diagnosed of IgA and IgG2 deficiency. Her IgG2 level of 1728 mg/L was still within the 95% range of the quoted reference range. Whether this is a case of genuine IgG2 deficiency or just sub-normal level of IgG2 warrants more thorough investigations after a proper reference interval for local population is setup.

Two patients and 5 control subjects have IgG3 levels lower than the set reference point. One of the 5 control subjects has an IgG3 level of 172 mg/L, which was out of the 95 percentile range. One patient was diagnosed of recurrent methicillin resistant Staphylococcus aureus infection. His IgG3 level was 15% lower than the reference point. Again the significance of this requires further investigations.

Nearly 80% of the adult patients studied suffered from bronchiectasis, which is defined as chronic dilatation of one or more bronchi [13]. De Gracia *et al.* [47] observed 39 abnormal low IgG2, IgG3 and IgG4 values among 31 out of 65 patients with bronchiectasis of no known cause. This is especially true of IgG2 deficiency, which accounts for 25 of the 39 abnormal low IgG subclass results. Mann Whitney

U test on the IgG2 concentrations of our bronchiectasis patient group with our control group demonstrated no significance difference (p=0.16).

7.2 Paediatric patients

Based on the same reference point criteria, IgG subclass concentration of paediatric patients enrolled in the study was compared with The Binding Site paediatric reference intervals.

There was one patient with all four subclass concentrations in the review range. The concentrations are however still within the reference interval for Birmingham paediatric population. This patient also presented with a total IgM of 0.1 g/L, IgA of <0.07 g/L and IgG of 4.7 g/L. He at the age of 17 would be expected to have a mature immune system. These low values strongly suggest immunodeficiency of some kind. Whether the panhypogammaglobulinaemia is of primary origin or secondary to other causes definite needs further investigations.

Other than the one patient discussed above, paediatric IgG2, IgG3 and IgG4 concentrations are all in the unremarkable range. Three values of IgG1 between 5000 mg/L to 6000 mg/L interval fulfil the criteria for further evaluation. In view of The significance of these results are doubtful. They are selected out as a result of our caution in selecting of review criteria in the absence of a control group and local reference interval.

7.3 Recurrent infections and IgG subclass deficiency

There is a whole spectrum of causes to recurrent respiratory tract infections other than IgG subclass deficiency. Any diseases that compromise the patients' lung function in predispose patients to recurrent pulmonary infection, tuberculosis being the commoner example. The negative finding in our group of 53 patients is therfore not difficult to explain. Furthermore, another cause of recurrent infections was not

studied in this patient group, namely, specific antibody deficiency that can be present in the absence of an IgG subclass deficiency.

7.4 Summary

In the current study, no IgG subclass deficiency case was identified out of the 53 patients enrolled. The clinical usefulness of the assays however should not be undermined. In order to obtain a better perspective on the situation in Hong Kong, a survey into the prevalence of IgG subclass deficiency should be conducted.

IgG subclass deficiency is an easy disorder to manage once identified. The efficacy of immunoglobulin replacement therapy had been reported [18].

The IgG subclass assays evaluated in this study is easy to perform and reliable. However, from a budgetary resource point of view, the assay is costly. Reagent cost per panel of four subclasses amount to HK$200 let alone the cost in reagents consumed for calibration and controls.

In conclusion, the IgG subclass assay should be make available since effective treatment is available once diagnosed. Intravenous immunoglobulin replacement therapy though not without risk but improve the quality of life of the patients and recover large number of productive hours as the severity of recurrent infections associated with IgG subclass deficiency may require home rest or even hospitalisation.

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